



(19) Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) EP 0 515 460 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
22.04.1998 Bulletin 1998/17

(21) Application number: 91903945.3

(22) Date of filing: 04.02.1991

(51) Int. Cl.⁶: A23L 1/054, A23L 1/30,
A61K 47/00, A23D 9/00,
A23D 9/02, A23D 9/04,
A23K 1/00, A23K 1/18,
C12P 7/64, C12N 1/13,
C12R 1/89, C11B 1/10,
A23K 1/16

(86) International application number:
PCT/US91/00733

(87) International publication number:
WO 91/11918 (22.08.1991 Gazette 1991/19)

(54) DOCOSAHEXAENOIC ACID, METHODS FOR ITS PRODUCTION AND COMPOUNDS CONTAINING THE SAME

DOCOSAHEXAENSÄURE, VERFAHREN ZU IHRER HERSTELLUNG UND SIE ENTHALTENDE
VERBINDUNGEN

ACIDE DOCOSAHEXAENOIQUE, SES PROCEDES DE PRODUCTION ET COMPOSES LE
CONTENANT

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(30) Priority: 13.02.1990 US 479135

(43) Date of publication of application:
02.12.1992 Bulletin 1992/49

(73) Proprietor: MARTEK CORPORATION
Columbia, MD 21045 (US)

(72) Inventors:
• KYLE, David, John
Catonsville, MD 21228 (US)
• REEB, Sue, Ellen
Baltimore, MD 21214 (US)
• SICOTTE, Valerie, Jacqueline
Baltimore, MD 21229 (US)

(74) Representative:
Marchant, James Ian et al
Elkington and Fife,
Prospect House,
8 Pembroke Road
Sevenoaks, Kent TN13 1XR (GB)

(56) References cited:
EP-A- 231 904 US-A- 4 868 001
US-A- 4 874 629 US-A- 4 911 944
US-A- 4 960 795 US-A- 4 963 385

- BIOCHEMICA ET BIOPHYSICA ACTA, Volume 712, No. 3, Issued 1982, U. Sonnenborn et al., "Purification and properties of the Fatty Acid Synthetase Complex from the Marine Dinoflagellate, Cryptocodium cohnii", pages 523-534
- CHEMICAL ABSTRACTS, Volume 100, Issued 1984, Karne et al., "Use of Fish Oil Fatty Acids (EPA and DHA) in Nutrition-Supplementing Foods and Drugs", see page 472, column 2, abstract no. 66638d, & Yushl, (1983), 36(12), 61-8.
- AMERICAN JOURNAL OF CLINICAL NUTRITION, Volume 51, No. 6, Issued 1990, S.M. Innis et al., "Plasma and Red Blood Cell Fatty Acids of Low-Birth-Weight Infants Fed Their Mother's Expressed Breast Milk or Preterm-Infant Formula" pages 994-1000

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 0 515 460 B1

Printed by Xerox (UK) Business Services
2 16 1/3 4

(Cont. next page)

EP 0 515 460 B1

• CHEMICAL ABSTRACTS, Volume 114, N . 5,
Issued 04 February 1991, NOGUCHI et al.,
"Feeds Containing Highly Unsaturated Fatty
Acids and Vitamin F for Swine and Production

of Pork Using th Feeds", see page 630,
column 1, Abstract No. 41472w; & JP,A,02 257
835

DescriptionBackground of the Invention

This invention relates to edible, single-cell oil containing docosahexaenoic acid (DHA). The invention also relates to methods of producing such oil containing DHA in commercially viable yields and to products containing the oil.

DHA is an omega-3-fatty acid and is the most abundant long chain polyunsaturated fatty acid (PUFA) in the grey matter of the brain. Omega-3-fatty acids in general are known to be beneficial in reducing the incidence of coronary heart disease [Lands, *Fish and Human Health* (1986) Academic Press]. However, the metabolism of omega-3-fatty acids is not well understood. Thus, precise clinical dosages and efficacy remain unknown.

Cold water marine fish are a known source of omega-3-fatty acids, including DHA. U.S. Patent No. 4,670,285 discloses the use of fish oil from fish such as menhaden and herring as a source of C₂₂ omega-3-fatty acids. Indeed, fish oils are the primary commercial source of omega-3-fatty acids. Often, however, fish oils are unusable for human consumption because of contamination with environmental pollutants such as PCB's.

There also are problems associated with the recovery of fish oils containing DHA for food uses. Such oils often have a fishy odor and unpleasant tastes associated with the oxidation products of the fatty acids. These tastes and toxicities of peroxides render the oils unsatisfactory for use in edible compositions such as baby food and infant formulas.

Marine microorganisms also are known to contain DHA. In particular, various species of dinoflagellates are known to contain DHA. Harrington et al., "The Polyunsaturated Fatty Acids of Marine Dinoflagellates" J. Protozool. 17:213-219 (1970), characterize the fatty acid content of eight photosynthetic and one heterotrophic marine dinoflagellates, and conclude that the dinoflagellates are a primary producer group of docosahexaenoic acid and contribute substantial amounts of that compound to the marine food chain.

Successful cultivation of dinoflagellates to produce an edible oil containing DHA has not been achieved. Dinoflagellates in general are very slow growing and are shear sensitive. Guillard et al., *Dinoflagellates*, (1984) Academic Press. The prior art discloses that even a small amount of agitation in the culturing vessel reduces growth of the cultures. However, such agitation would be necessary to achieve adequate oxygenation in order to maximize growth for commercial production.

DHA is thought to be essential for the proper brain and vision development of infants because, as noted above, it is the most abundant long chain PUFA in the brain and retina. Although a metabolic pathway exists in mammals for the biosynthesis of DHA from dietary linoleic acid, this pathway is bioenergetically unfavorable

[Crawford, P. *AOCS Short Course in Polyunsaturated Fatty Acids and Eicosanoids*, pp. 270-295 (1987)] and mammals, like fish, are thought to obtain most of their DHA from dietary sources. In the case of infants, the most likely source would be human milk. Indeed, DHA is the most abundant C20 omega-3 PUFA in human milk. Generally, however, DHA is absent from infant formulas. U.S. Patent No. 4,670,285 does disclose an infant formula containing omega-3-fatty acids. However, the acids utilized therein are obtained from egg or fish (Talapia) oil and have associated therewith the unpleasant characteristics previously described. Furthermore, fish oils generally contain another omega-3-fatty acid, eicosapentaenoic acid (EPA), an undesirable component in infant formulas because of its prolonged anticoagulant effects and its depression of arachidonic levels in infants. This has been correlated with reduced rates of infant weight gain (Carleson et al. INFORM 1:306.) Indeed, EPA levels are very low in human milk (less than one-fourth that of DHA).

Accordingly, it is an object of the present invention to provide a single-cell edible oil containing DHA. Preferably this oil will have no significant quantities of other polyunsaturated fatty acids (PUFA's), i.e. greater than about 2% of the total fatty acid content. In general, it is an object of the present invention to produce single-cell oil in commercially viable yields.

Accordingly the present invention provides a single cell-edible oil characterized in that docosahexaenoic acid (DHA) makes up at least 15% of oil, by weight, preferably at least 20%, more preferably 30% and most preferably 35%.

The present invention also provides a method of producing a single cell-edible oil as defined above comprising:

cultivating a microorganism capable of producing said single cell oil in a fermentor to achieve a cell density of at least about 10 grams biomass per liter of a nutrient solution,
wherein the microorganism is a dinoflagellate and further wherein the microorganism is induced, by the imposition of a stationary phase, to produce the single cell oil at a concentration of at least about 1.5 grams per liter of nutrient solution, and
harvesting the biomass and recovering the single cell oil from the biomass oil.

The oil, characterized herein as a "designer" oil, after extraction can be used in infant formulas, baby foods, dietary supplements and pharmaceuticals.

In addition, it would be desirable to acquire further knowledge of the metabolic pathway of omega-3 fatty acids. Isotopically labelled DHA would be of great utility in this regard. However, to date, no method has been known to produce abundant quantities of isotopically labeled DHA. Thus, it also is an object of the present invention to provide isotopically labeled DHA in suffi-

cient quantities to undertake such research.

Summary of the Invention

The present invention relates to the cultivation of microorganisms, notably dinoflagellates, in a fermentor, induction of those microorganisms to produce significant quantities of single cell oil containing a high proportion of DHA and recovery of that oil. As used herein, "single cell oil" refers to a lipid product of a unicellular organism. The present invention also includes mutant organisms capable of producing enhanced quantities of single-cell oil containing at least about 20% by weight DHA and includes single cell oil containing DHA.

The present invention provides an economical method of obtaining enhanced levels of edible oils containing DHA. Additionally, the method permits the commercial cultivation of dinoflagellates in elevated cell densities.

Edible oils produced by the method of this invention lack unpleasant tastes and fishy odors and also are free of environmental contaminants often found in DHA-containing oils from conventional sources. Accordingly, the present invention further includes food products containing the oil of this invention.

Brief Description of the Drawings

Figures 1, 2 and 3 are graphic illustrations of *C. cohnii* biomass accumulation over time with the addition of various nutrients.

Detailed Description of the Best Mode Of Practicing the Invention

In accordance with the present invention, microorganisms capable of producing a single cell oil containing DHA are cultivated in a fermentor in a nutrient solution capable of supporting the growth of such organisms. Preferably the single cell oil will contain at least about 20% by weight DHA.

Any microorganisms capable of producing a single-cell edible oil containing DHA can be used in the present invention. For example, photosynthetic diatoms can be used. Preferred microorganisms are marine dinoflagellates, including *Cryptocodinium* sp. Especially preferred is *Cryptocodinium cohnii*, an obligate heterotroph requiring a reduced carbon source for growth. *C. cohnii* is preferred because it contains a fatty acid profile in which DHA is the only PUFA present in sufficient quantities (greater than about 1% of the total amount of PUFAs). Samples of this organism, designated MK8840, have been deposited with the American Type Culture Collection at Rockville, Maryland, and assigned accession number 40750. As used herein, microorganism, or any specific type of microorganism, includes wild strains, mutants or recombinant types. Any microorganism which produces enhanced levels of

oil containing DHA is considered to be within the scope of this invention. One of the features of the present invention is its recognition of the edible oil-producing capability of microorganisms such as dinoflagellates and the attendant solution to the problem of maintaining a reliable, economic source of such oils. Accordingly, wild-type and recombinant microorganisms designed to produce single cell oil containing DHA are an aspect of this invention. Such recombinant organisms would include those designed to produce greater quantities of DHA in the single cell oil, greater quantities of total oil, or both, as compared to the quantities produced by the same wild type microorganism, when provided with the same substrates. Also included would be microorganisms designed to efficiently use more cost-effective substrates while producing the same amount of single cell oil containing DHA as the comparable wild-type microorganism.

In general, those of skill in the art would not consider *C. cohnii* a suitable organism for cultivation in a fermentor. Previous workers have commented on the extremely complex mixture of nutrients required to successfully cultivate *C. cohnii*. Gold et al. *Protozoal*, 13:255-257 (1966); Guillard, et al. in "Dinoflagellates", Academic Press (1984); Henderson, et al., *Phytochemistry* 27:1679-1683 (1988). In contrast, the present invention achieves the cultivation of DHA-producing microorganisms in a simple medium containing glucose and yeast extract. Use of these components in a solution such as seawater provides economically significant growth rates and cell densities. For example, during the course of a 3-5 day fermentation, *C. cohnii* cell densities of at least 10 grams of biomass per liter of solution, and typically from 20 to about 40 grams per liter, can be attained. Such densities have not heretofore been attainable.

Although cultivation can occur in any suitable fermentor, preferably the organism is grown either in a stirred tank fermentor (STF) or in an air lift fermentor (ALF), both types known to those of skill in the art. When a STF is selected, agitation is provided using either Rushton-type high efficiency turbines or pitched-blade or marine impellers. Agitation and sparging renew the supply of oxygen to the microorganisms. The rate of agitation normally is increased as the biomass increases, due to the increased demand for oxygen. It is desirable to keep the tip speed at not greater than about 500 cm/sec, preferably not greater than about 300 cm/sec. Selection of strains of microorganisms which are capable of withstanding greater tip speeds without undergoing shear is within the purview of those of skill in the art. The use of such strains is expressly included in this invention.

As noted above, seawater is an acceptable medium for the nutrient solution. The seawater can be either natural, filtered or an artificial mix, each of which can be diluted to reduced salinities, such as 1/2 to 1/4 normal strength, with tap water or concentrated to 2 times nor-

mal strength. A preferred example is Instant Ocean® (IO) brand artificial seawater. Although *C. cohnii* is a marine microorganism, some growth has been observed in zero salinity. The use of variants which grow well in reduced salinities is specifically encompassed by this invention. Micronutrients can be added and may be required at low salinities. However, such micronutrients are known to those of skill in the art and generally are present in seawater or tap water. If the organism selected is heterotrophic, such as *C. cohnii*, then a carbon source is added.

Preferably, after addition of the seawater medium to the fermentor, the fermentor containing the medium is sterilized and cooled prior to adding the nutrients and a seeding population of microorganism. (Although it is acceptable to sterilize the nutrients together with the seawater, sterilization in this manner can result in a loss of available glucose.) The nutrients and microorganism can be added simultaneously or sequentially.

An effective seed concentration can be determined by those of skill in the art. When a STF is used, the addition of a population of from about .05 to 1.0 grams of dry weight equivalent per liter at the beginning of the fermentation is preferred. This is about 10^6 cells per ml. Thus, for a 30 liter fermentor, 1-3 liters of seeding media, containing viable cells at a density of 20g dry weight per liter would be added.

Oxygen levels preferably are maintained at a D.O. of at least about 10% of air saturation level. Biosynthesis of DHA requires oxygen and, accordingly, higher yields of DHA require D.O. levels at from about 10% to 50% of air saturation levels. Agitation tip speeds of 150-200 cm/sec in combination with an aeration rate of 1 VVM (volume of air/volume of fermentor per minute) provides D.O. levels of from about 20% to about 30% at biomass densities of about 25 g dry weight/liter of culture. Higher cell densities may require higher D.O. levels, which can be attained by increased aeration rates by O₂ sparging, or by increasing the air pressure in the fermentor.

Acceptable carbon sources are known to those of skill in the art. For example, carbon can be provided to *C. cohnii* in the form of glucose. Other heterotrophs can use other reduced carbon sources, a matter easily determined by those of skill in the art, and autotrophs utilize carbon dioxide. *C. cohnii* will also grow on other reduced, more complex, carbon sources. Typically, a fermentation is initiated with about 10-50 g/liter glucose. More glucose is added during the fermentation as required. Alternatively, from about 50 to 150 g, preferably 50 to 100g glucose/liter initially can be added, thereby minimizing the frequency of future additions. The amount of carbon source provided to other organisms can readily be determined by those of skill in the art.

In addition to a reduced carbon source, a nitrogen source, such as yeast extract (YE), is provided to the medium. Commercially available yeast extract is

acceptable. For example, DIFCO or MARCOR brand yeast extract can be used. The yeast extract is an organic nitrogen source also containing micronutrients. Other organic nitrogen sources easily can be determined by those of skill in the art. However, such compounds are generally more expensive than yeast extract.

- 5 Other organic nitrogen sources easily can be determined by those of skill in the art. However, such compounds are generally more expensive than yeast extract. The use of variants capable of growing on urea or nitrates is within the scope of this invention. Typically, the fermentation is initiated with about 6-12 g YE/liter.
- 10 More YE can be added as required. A typical fermentation run requires from about 8 to 15 g YE/liter over the course of the run. Accordingly, that amount of YE can be added initially with a reduced need for further additions. The precise amount can be determined by those of skill in the art. Generally, the ratio of glucose to YE is from about 2:1 to about 15:1.
- 15

The cultivation can be carried out at any life-sustaining temperature. Generally *C. cohnii* will grow at temperatures ranging from about 15°C to 34°C. Preferably the temperature is maintained at about 20-30°C.

- 20 Strains which grow at higher temperatures are preferred, because they will have a faster doubling time, thereby reducing the fermentation time. Appropriate temperature ranges for other microorganisms are readily determined by those of skill in the art.
- 25

The cultivation can be carried out over a broad pH range, typically from about pH 5.0 to 9.0. Preferably, a pH range of from about 6.0 to about 7.0 is used for the growth phase. A base, such as KOH or NaOH, is used to adjust the media pH prior to inoculation. During the later stages of the fermentation, the culture medium tends to become alkaline. If desired, inorganic acid pH controls can be used to correct alkalinity during the growth phase.

- 30
- 35 Production of the single cell oil is induced in the dinoflagellates by the imposition of a stationary phase (i.e., by nitrogen depletion or a pH rise). YE deficiencies are caused by providing YE in a limiting amount such that the medium runs out of YE while available glucose remains. The present invention recognizes that it is the carbon source to nitrogen source ratio which promotes the efficient production of the single cell oil. Using glucose and YE as exemplary, a preferred ratio of carbon source to nitrogen source is about 10-15 parts glucose to 1 part YE. Similar ratios for other carbon and nitrogen sources can be calculated by those of skill in the art.
- 40
- 45

After induction of oil production, the culture is grown for about 24 additional hours. During this period of oleosynthesis, the single cell oil containing DHA is being synthesized and visible oil droplets become apparent. Those of skill in the art can readily calculate the time of fermentation required to achieve the expected amount of cell biomass based upon the added amount of YE. When that time has passed, the culture is grown for an additional 24 hours and harvested. In general, the *C. cohnii* are cultivated for a time sufficient to produce single cell oil, usually from about 60 to about 90 hours, although this time is subject to variation.

From about 15 to 30% of the resultant biomass, using wild-type *C. cohnii*, comprises extractable oil. Strain selection can increase this percentage and such selection is within the scope of this invention. Preferably, the oil comprises greater than about 70% triglycerides having, in general, the following fatty acid composition.

15-20% myristic acid (C_{14:0})
 20-25% palmitic acid (C_{16:0})
 10-15% oleic acid (C_{18:1})
 30-40% DHA (C_{22:6})
 0-10% others

(Other oil components including polar lipids, such as phosphatidyl choline, also may be enriched in DNA.) The crude oil is characterized by a yellow-orange color and is liquid at room temperature. Desirably, the oil contains at least about 20% DHA by weight and most preferably at least about 35% DHA by weight.

The organisms are harvested by conventional means, known to those of skill in the art, such as centrifugation, flocculation or filtration, and can be processed immediately or dried for future processing. In either event, the oil can be extracted readily with an effective amount of solvent. Suitable solvents can be determined by those of skill in the art. However, preferred solvents include pure hexane and supercritical fluids, such as supercritical CO₂.

Extraction techniques using supercritical fluids are known to those of skill in the art and described in McHugh et al., *Supercritical Fluid Extraction*, Butterworth, 1986. If the extraction solvent is hexane, a suitable ratio of hexane to dry biomass is about 4 liters of hexane per kilogram of dry biomass. The hexane preferably is mixed with the biomass in a stirred reaction vessel at a temperature of about 20-50°C for about 2 hours. After mixing, the biomass is filtered and separated from the hexane containing the oil. Alternatively, a wet biomass paste (30-35% solids) can be extracted directly with more polar solvents, such as ethanol, isopropanol or hexane/isopropanol mixtures. The residual biomass, i.e. the single cell edible oil extracted biomass of the microorganisms, such as *C. cohnii*, can be used as an animal feed, containing as it does about 35-40% protein, 8-10% ash and 45-50% carbohydrates. Because of this high protein content and the elevated levels of DHA, the whole biomass paste can be used for aquaculture (e.g., shrimp, oysters, fish) feed.

The solvent then is removed from the oil by distillation techniques known to those of skill in the art. Conventional oilseed processing equipment is suitable to perform the filtering, separation and distillation. Additional processing steps, known to those of skill in the art, can be performed if required or desirable for a particular application. These steps also will be similar to those involved in conventional vegetable oil processing and allow the separation of DHA-enriched polar lipid fractions.

5 Isotopically labeled single cell oils, including labeled DHA, can be easily obtained in sufficient quantities to permit research into the metabolic pathways of DHA by the method of this invention. When ¹³C-glucose or ¹⁴C-glucose is provided as the reduced carbon substrate, labeled DHA results.

10 The present invention also includes food products, such as infant formulas and baby foods, as well as dietary supplements, which contain the single-cell oil containing DHA of the present invention. While those of skill in the art have recognized that infant formulas containing DHA are desirable, the prior art infant formulas contained DHA from fish oil, with its attendant unpleasant tastes and organoleptic characteristics. Furthermore, fish oil supplementation of infant formula includes the addition of eicosapentaenoic acid (EPA), an omega-3-fatty acid known to possess anticoagulant activity and possibly responsible for reduction of arachidonic acid biosynthesis. Such an activity is not desirable in infant formula or baby food and the single cell oil described herein contains no significant quantity of EPA. Food products, such as infant formula, containing the single cell oil of the present invention do not have the unpleasant organoleptic characteristics of fish oil. The food products thus are more readily accepted by infants and adults alike. Preferably the infant formula of the present invention contains about 0.05% by weight of single cell oil containing DHA. The baby food of the present invention, having a more solid constitution, preferably contains about 0.5% by weight of single cell oil containing DHA. In both instances, most preferably, the oil contains at least about 35% DHA.

15 The present invention includes pharmaceutical products including single cell oil containing DHA. Preferably the products contain at least about 35% DHA. Exemplary of such pharmaceutical products is one suitable for use in providing total parenteral nutrition (TPN) to infants or adults. Additionally, dietary supplements containing the single cell oil are encompassed. Preferably, such supplements are in the form of gelatin capsules encapsulating said oil and may be appropriate for pregnant women or breast feeding mothers. This especially may be true for such women who are vegetarians and do not get sufficient amounts of DHA in their diets.

20 The present invention also includes single cell oil containing DHA. Preferably the single cell oil contains at least about 20% by weight DHA. Most preferably the oil contains at least about 35% by weight DHA.

25 The present invention having been generally described, reference is had to the following non-limiting specific examples.

Example 1

30 Into a 30-liter working volume STF was loaded a medium of one half strength artificial seawater. Six liters of IO were combined with 18 liters of tap water. The fermentor containing the medium was sterilized and

cooled to 28°C. Four hundred ml of concentrated YE (455g/l), 900 ml of glucose syrup (400 g/l) and one liter of inoculum from a seed fermentor containing about 2×10^7 cells/ml or a biomass of 20 g/liter (yielding a final concentration of about 7×10^6 cells/ml or a biomass of about 700 mg/liter), were added to the medium. Agitation was set at 120 cm/sec tip speed and aeration was set at 1 VVM (30 liters per minute). Additional glucose syrup (900 ml) was added after 30 hours and another 4.2 liters over the next 42 hours. Thus 6 liters of glucose syrup were added in total. Concentrated YE solution (400 ml) was added at hour 6 and another 1.2 liters were added over the next 48 hours until a total of 2.0 liters had been added. To maintain the D.O. at greater than 20%, at 24 hours the agitation tip speed was increased to 150 cm/sec and at 48 hours to 160 cm/sec. At 72 hours, the tip speed was increased to 200 cm/sec and the culture was permitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culturing conditions are depicted graphically in Figure 1. The culture was then harvested by centrifugation with the cell pellet retained. The harvested pellet of cells was frozen and dried (lyophilized) to about a 4% moisture content. Hexane (2.8 liters) was added to the dried biomass and stirred in a glass kettle for 1.5 hours at 50°C. A rotary evaporator was used to remove the hexane, producing about 175 g of crude DHA-containing oil.

Example 2

Into a 350-liter working volume STF was loaded a medium of one half strength artificial seawater made by combining 4.3 kg. of I.O.® with 230 liters of tap water. The fermenter containing the medium was sterilized and cooled to 28°C. 6.8 liters of concentrated YE (400g/l), 12.5 liters of glucose syrup (400g/l) and 30 liters of *C. cohnii* inoculum from a seed fermenter (10^6 cells/ml or a biomass density of about 1.3g/liter) were added to the medium. Agitation was set at 73 cm/sec tip speed and aeration was set at 1 VVM (280 liters per minute). Additional glucose syrup (12 liters) was added after about 44 hours and another 43 liters over the next 32 hours. Thus, 67.5 liters of glucose syrup were added in total. The glucose additions and the cell growth are depicted graphically in Figure 2.

To maintain the D.O. at greater than 20%, at 44 hours the agitation tip speed was increased to 175 cm/sec and at 55 hours to 225 cm/sec. At 76 hours, the tip speed was decreased to 150 cm/sec and the culture was permitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culture then was harvested. The harvested cells were dried to about a 4% moisture content. Hexane was added to the dried biomass and stirred in a glass kettle for 2 hours at 25°C. A rotary evaporator was used to remove the hexane, producing about 700 g of crude DHA-containing oil.

Example 3

Into a 30-liter working volume STF was loaded a medium of full strength artificial seawater made by combining 565g of I.O.® with 15 liters of tap water. The fermenter containing the medium was sterilized and cooled to 28°C. Four hundred ml of concentrated YE (400g/l), 1.9 liters of glucose syrup (400g/l) and 1 liter of *C. cohnii* inoculum from a seed fermenter (10^6 cells/ml or a biomass of about 2.0g/liter) were added to the medium. Agitation was set at 80 cm/sec tip speed and aeration was set at 1 VVM (20 liters per minute). Additional glucose syrup (1.5 l) was added after 94 hours and another 1.1 liters at 116 hours. Thus 4.5 liters of glucose syrup were added in total. To maintain the D.O. at greater than 20%, at 52 hours the agitation tip speed was increased to 160 cm/sec. At 66 hours, stationary phase was induced and in order to accomplish this, the pH was spiked with 4N KOH to 7.0 and the agitation tip speed was not further increased for the duration of the run. As shown in Figure 3, the culture was permitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culture then was harvested. The harvested cells were dried to about a 4% moisture content. Hexane was added to the dried biomass and stirred in a glass kettle for 1.5 hours at 50°C. A rotary evaporator was used to remove the hexane, producing about 65 g of crude DHA-containing oil.

Claims

1. A single cell-edible oil characterized in that docosahexaenoic acid (DHA) makes up at least 15% of the oil by weight, preferably at least 20%, more preferably at least 30% and most preferably at least 35%.
2. An oil as claimed in claim 1, which does not contain any significant quantity of EPA.
3. An oil as claimed in claim 1 or 2, which is obtained from a dinoflagellate, preferably *Cryptocodinium* sp., more preferably *Cryptocodinium cohnii*.
4. A method of producing a single cell edible oil as claimed in any of claims 1 to 3, by cultivating a microorganism capable of producing the said single cell oil in a fermentor to achieve a cell density of at least about 10 grams biomass per liter of a nutrient solution, harvesting the biomass and recovering the single cell oil from the biomass, wherein the microorganism is a dinoflagellate and the microorganism is induced to produce the single cell oil at a concentration of at least about 1.5 grams per liter of nutrient solution by imposition of a stationary phase.
5. A method as claimed in claim 4, wherein the microorganism is of the genus *Cryptocodinium*, preferably *Cryptocodinium cohnii*.

6. A method as claimed in claim 4 or 5, wherein the nutrient solution comprises seawater or artificial seawater. 5
7. A method as claimed in claim 6, wherein the nutrient solution comprises reduced salinity seawater or reduced salinity artificial seawater. 10
8. A method as claimed in any of claims 4 to 7, wherein the nutrient solution comprises a reduced carbon source and an organic nitrogen source. 15
9. A method as claimed in claim 8, wherein the ratio of the reduced carbon source to the organic nitrogen source is equivalent to a ratio of glucose to yeast extract of from about 2 to about 15 parts glucose to 1 part yeast extract. 20
10. A method as claimed in any of claims 4 to 9, further comprising maintaining a dissolved oxygen level of at least about 10% of air saturation substantially throughout the fermentation. 25
11. A method as claimed in any of claims 4 to 9, wherein the microorganism is cultivated to a cell density of at least about 20 grams of biomass per liter of nutrient solution. 30
12. A method as claimed in any of claims 4 to 11 for the production of a single cell-edible oil containing at least 20% DHA by weight, said method comprising: 35
- (a) adding about 10^6 cells/ml (0.5-1.0 g dry wt/liter) of *C. cohnii* to a fermentor initially containing a nutrient solution comprising about one-quarter to one-half strength artificial seawater, 1.8% glucose and 0.4-0.8% yeast extract; 40
 - (b) cultivating said *C. cohnii* at a temperature of from about 15°C to about 34°C and a pH of from about 5.0 to 9.0;
 - (c) incrementally adding glucose and yeast extract to said nutrient solution for about 56 hours;
 - (d) adding further glucose to said nutrient solution for about 16 additional hours to induce said *C. cohnii* to produce oil; 45
 - (e) maintaining a dissolved oxygen content of at least about 20% of air saturation level throughout said cultivation;
 - (f) harvesting said *C. cohnii* after about 60 to 90 hours; and
 - (g) recovering the single-cell edible oil. 50
13. A food product or pharmaceutical product containing a single cell-edible oil as claimed in any of claims 1 to 3 or as produced by a method as claimed in any of claims 4 to 12. 55
14. A food product as claimed in claim 13 which is an infant formula, a baby food or a dietary supplement.
15. A pharmaceutical product as claimed in claim 13, which is suitable for use in providing total parenteral nutrition or as a dietary supplement.
16. An animal feed comprising a biomass of microorganisms cultivated by a method as claimed in any of claims 4 to 12.
17. An animal feed as claimed in claim 16, wherein the biomass has been treated with a solvent such that at least some of the oil is extracted into the solvent.
18. An animal feed as claimed in claim 16 or 17 which is an aquaculture feed.
19. Use of a microbial biomass as an aquaculture feed, the biomass having been produced by cultivating a microorganism capable of producing a single cell oil in which DHA makes up at least 15% of the oil by weight in a fermentor to achieve a cell density of at least about 10 grams biomass per liter of nutrient solution and harvesting the biomass, wherein the microorganism is a dinoflagellate and the microorganism is induced to produce the single cell oil at a concentration of at least about 1.5 grams per liter of nutrient solution by imposition of a stationary phase. 60
20. Use as claimed in claim 19, wherein the aquaculture comprises shrimp, oysters or fish.
21. Use of a single cell oil as claimed in any of claims 1 to 3 or as produced by a method as claimed in any of claims 4 to 12 in the preparation of a food product, a product suitable for providing total parenteral nutrition or a product suitable as a dietary supplement. 65
22. Use as claimed in claim 21, wherein the food product is an infant formula, a baby food or a dietary supplement.
- Patentansprüche**
1. Speiseöl von Einzellern, dadurch gekennzeichnet, daß Docosahexaensäure (DHA) mindestens 15 Gew.-% des Öls ausmacht, vorzugsweise mindestens 20 %, mehr bevorzugt mindestens 30 % und am meisten bevorzugt mindestens 35 %.
 2. Öl nach Anspruch 1, das keine signifikanten Mengen EPA enthält.
 3. Öl nach Anspruch 1 oder 2, das von einem Dinoflagellaten erhalten wird, vorzugsweise Cryptocodi-

- nium sp., mehr bevorzugt Cryptecodinium cohnii.
4. Verfahren zur Herstellung eines Speiseöls von Einzellen nach einem der Ansprüche 1 bis 3, wobei man einen Mikroorganismus, der zur Produktion des Öls von Einzellern fähig ist, in einem Fermenter züchtet, bis man eine Zeldichte von mindestens etwa 10 g Biomasse pro Liter Nährstofflösung erhält, die Biomasse erntet und das Öl von Einzellern aus der Biomasse gewinnt, wobei der Mikroorganismus ein Dinoflagellat ist und der Mikroorganismus zur Produktion des Öls von Einzellern in einer Konzentration von mindestens etwa 1,5 g pro Liter Nährstofflösung durch Auslösen einer stationären Phase veranlaßt wird.
5. Verfahren nach Anspruch 4, wobei der Mikroorganismus von der Gattung Cryptecodinium stammt, vorzugsweise Cryptecodinium cohnii.
6. Verfahren nach Anspruch 4 oder 5, wobei die Nährstofflösung Meerwasser oder künstliches Meerwasser umfaßt.
7. Verfahren nach Anspruch 6, wobei die Nährstofflösung im Salzgehalt reduziertes Meerwasser oder im Salzgehalt reduziertes künstliches Meerwasser umfaßt.
8. Verfahren nach einem der Ansprüche 4 bis 7, wobei die Nährstofflösung eine verminderte Kohlenstoffquelle und eine organische Stickstoffquelle umfaßt.
9. Verfahren nach Anspruch 8, wobei das Verhältnis der verminderten Kohlenstoffquelle zur organischen Stickstoffquelle äquivalent ist zu einem Verhältnis von Glucose zu Hefeextrakt von etwa 2 bis etwa 15 Teilen Glucose zu 1 Teil Hefeextrakt.
10. Verfahren nach einem der Ansprüche 4 bis 9, weiterhin umfassend die Aufrechterhaltung eines Spiegels von gelöstem Sauerstoff von mindestens etwa 10 % Luftsättigung, im wesentlichen während der ganzen Fermentation.
11. Verfahren nach einem der Ansprüche 4 bis 9, wobei der Mikroorganismus bis zu einer Zeldichte von mindestens etwa 20 g Biomasse pro Liter Nährstofflösung gezüchtet wird.
12. Verfahren nach einem der Ansprüche 4 bis 11 zur Produktion eines Speiseöls von Einzellern, enthaltend mindestens 20 Gew.-% DHA, wobei das Verfahren folgende Schritte umfaßt:
- (a) Eingabe von 10^6 Zellen/ml (0,5 - 1,0 g Trockengewicht/Liter) C. cohnii in einen Fermenter, der zunächst eine Nährstofflösung enthält, die
- 5 10 15 20 25 30 35 40 45 50 55
- künstliches Meerwasser mit etwa 1/4 bis 1/2 der Konzentration, 1 bis 8 % Glucose und 0,4 bis 0,8 % Hefeextrakt enthält;
- (b) Züchtung des C. cohnii bei einer Temperatur von etwa 15°C bis etwa 34°C und einem pH-Wert von etwa 5,0 bis 9,0;
- (c) mehrfache Zugabe von Glucose und Hefeextrakt zu der Nährstofflösung für etwa 56 Stunden;
- (d) weitere Zugabe von Glucose zu der Nährstofflösung für etwa 16 weitere Stunden, um C. cohnii zur Produktion von Öl zu veranlassen;
- (e) Aufrechterhaltung eines Gehalts an gelöstem Sauerstoff von mindestens etwa 20 % Luftsättigung während der ganzen Züchtung;
- (f) Ernten von C. cohnii nach etwa 60 bis 90 Stunden; und
- (g) Gewinnung des Speiseöls von Einzellern.
13. Nahrungsmittel oder Arzneimittel, das ein Speiseöl von Einzellern nach einem der Ansprüche 1 bis 3 enthält oder das nach einem Verfahren nach einem der Ansprüche 4 bis 12 hergestellt wurde.
14. Nahrungsmittel nach Anspruch 13, das eine künstliche Säuglingsnahrung oder Babynahrung oder ein Nahrungsergänzungsmittel ist.
15. Arzneimittel nach Anspruch 13, das zur Verwendung für die Bereitstellung vollständiger parenteraler Ernährung oder als ein Nahrungsergänzungsmittel geeignet ist.
16. Tiernahrung, umfassend eine Biomasse von Mikroorganismen, die gemäß einem Verfahren nach einem der Ansprüche 4 bis 12 gezüchtet wurden.
17. Tiernahrung nach Anspruch 16, wobei die Biomasse mit einem Lösungsmittel behandelt wurde, so daß zumindest ein Teil des Öls in das Lösungsmittel extrahiert wurde.
18. Tiernahrung nach Anspruch 16 oder 17, die ein Aquakulturnährmittel ist.
19. Verwendung einer mikrobiellen Biomasse als Aquakulturnährmittel, wobei die Biomasse durch Züchtung eines Mikroorganismus in einem Fermenter produziert wurde, der fähig ist zur Produktion eines Öls in Einzellern, in dem die DHA mindestens 15 Gew.-% des Öls ausmacht, um eine Zeldichte von mindestens etwa 10 g Biomasse pro Liter Nährstofflösung zu erzielen, und durch Ernten der Biomasse, wobei der Mikroorganismus ein Dinoflagellat ist und der Mikroorganismus zur Produktion des Öls von Einzellern in einer Konzentration von mindestens etwa 1,5 g pro Liter Nährstofflösung durch Auslösen einer stationären

Phase veranlaßt wird.

20. Verwendung nach Anspruch 19, wobei die Aquakultur Garnelen, Austern oder Fisch umfaßt.

21. Verwendung eines Öls aus Einzellern nach einem der Ansprüche 1 bis 3 oder hergestellt gemäß einem Verfahren nach einem der Ansprüche 4 bis 12 für die Herstellung eines Nahrungsmittels, eines Produkts, das zur Bereitstellung vollständiger parenteraler Ernährung geeignet ist, oder eines Produkts, das als Nahrungsergänzungsmittel geeignet ist.

22. Verwendung nach Anspruch 21, wobei das Nahrungsmittel eine künstliche Säuglingsnahrung, Babynahrung oder ein Nahrungsergänzungsmittel ist.

Revendications

1.. Huile comestible d'unicellulaire caractérisée en ce que l'acide docosahexanoïque (DHA) constitue au moins 15% en poids de l'huile, de préférence au moins 20%, mieux encore au moins 30% et mieux encore au moins 35%.

2. Huile telle que revendiquée dans la revendication 1, qui ne contient aucune quantité significative d'EPA.

3. Huile telle que revendiquée dans la revendication 1 ou 2, qui est obtenue à partir d'un dinoflagellé, de préférence, *Cryptocodinium sp.*, mieux encore *Cryptocodinium cohnii*.

4. Méthode de production d'une huile comestible d'unicellulaire telle que revendiquée dans l'une quelconque des revendications 1 à 3, dans laquelle on cultive un micro-organisme capable de produire ladite huile d'unicellulaire dans un fermenteur, pour obtenir une densité de cellules d'au moins environ 10 grammes de biomasse par litre de solution nutritive, on récolte la biomasse et on récupère l'huile d'unicellulaire à partir de la biomasse, le micro-organisme étant un dinoflagellé et le micro-organisme étant induit à produire de l'huile d'unicellulaire à une concentration d'au moins environ 1,5 grammes par litre de solution nutritive par imposition d'une phase stationnaire.

5. Méthode telle que revendiquée dans la revendication 4, dans laquelle le micro-organisme appartient au genre *Cryptocodinium*, et est de préférence *Cryptocodinium cohnii*.

6. Méthode telle que revendiquée dans la revendication 4 ou la revendication 5, dans laquelle la solution nutritive comprend de l'eau de mer ou de l'eau

de mer artificielle.

7. Méthode telle que revendiquée dans la revendication 6, dans laquelle la solution nutritive comprend de l'eau de mer à salinité réduite ou de l'eau de mer artificielle à salinité réduite.

8. Méthode telle que revendiquée dans l'une quelconque des revendications 4 à 7, dans laquelle la solution nutritive comprend une source de carbone réduite et une source d'azote organique.

9. Méthode telle que revendiquée dans la revendication 8, dans laquelle le rapport de source de carbone réduite à la source d'azote organique est équivalent à un rapport de glucose à extrait de levure d'environ 2 à environ 15 parties de glucose à une partie d'extrait de levure.

10. Méthode telle que revendiquée dans l'une quelconque des revendications 4 à 9, comprenant en outre le maintien d'un taux d'oxygène dissous d'au moins environ 10% de saturation d'air essentiellement dans le mélange de fermentation.

11. Méthode telle que revendiquée dans l'une quelconque des revendications 4 à 9, dans laquelle les micro-organismes sont cultivés à une densité de cellules d'au moins environ 20 grammes de biomasse par litre de solution nutritive.

12. Méthode telle que revendiquée dans l'une quelconque des revendications 4 à 11, pour la production d'une huile comestible d'unicellulaire contenant au moins environ 20% en poids de DHA, ladite méthode comprenant les étapes consistant :

(a) à ajouter environ 10^6 cellules/ml (0,5-1,0 g pds sec/litre) de *C. cohnii* dans un fermenteur contenant initialement une solution nutritive comprenant de l'eau de mer artificielle d'une force d'environ un quart à un demi, 1-8% de glucose et 0,4-0,8% d'extrait de levure ;

(b) à cultiver le dit *C. cohnii* à une température d'environ 15°C à environ 34°C et à un pH d'environ 5,0 à 9,0 ;

(c) à ajouter progressivement du glucose et de l'extrait de levure à ladite solution nutritive pendant environ 56 heures ;

(d) à ajouter encore du glucose à ladite solution nutritive pendant environ 16 heures supplémentaires pour induire la production d'huile par *C. cohnii* ;

(e) à maintenir un taux d'oxygène dissous d'au moins environ 20% du taux de saturation de l'air pendant ladite culture ;

(f) à récolter ledit *C. cohnii* au bout d'environ 60 à 90 heures ; et

- (g) à récupérer l'huile comestible d'unicellulaire.
13. Produit alimentaire ou produit pharmaceutique contenant une huile comestible d'unicellulaire telle que revendiquée dans l'une quelconque des revendications 1 à 3 ou telle que produite par une méthode telle que revendiquée dans l'une quelconque des revendications 4 à 12.
14. Produit alimentaire tel que revendiqué dans la revendication 13, qui est une formule pour nourrisson, un aliment pour bébé ou un supplément diététique.
15. Produit pharmaceutique tel que revendiqué dans la revendication 13, qui convient à une utilisation dans l'apport d'une nutrition parentérale totale ou comme supplément diététique.
16. Aliment pour animaux comprenant une biomasse de micro-organismes cultivée par une méthode telle que revendiquée dans l'une quelconque des revendications 4 à 12.
17. Aliment pour animaux tel que revendiqué dans la revendication 16, dans lequel la biomasse a été traitée avec un solvant de telle sorte qu'au moins une certaine partie de l'huile est extraite dans le solvant.
18. Aliment pour animaux tel que revendiqué dans la revendication 16 ou la revendication 17, qui est un aliment pour aquaculture.
19. Utilisation d'une biomasse microbienne comme aliment pour aquaculture, la biomasse ayant été produite en cultivant un micro-organisme capable de produire une huile d'unicellulaire dans laquelle le DHA constitue au moins 15% en poids de l'huile, dans un fermenteur, pour obtenir une densité de cellules d'au moins environ 10 grammes de biomasse par litre d'une solution nutritive et en récoltant la biomasse, dans laquelle le micro-organisme est un dinoflagellé et dans laquelle on induit la production par le micro-organisme de l'huile d'unicellulaire à une concentration d'au moins environ 1,5 gramme par litre de solution nutritive en imposant une phase stationnaire.
20. Utilisation telle que revendiquée dans la revendication 19, dans laquelle l'aquaculture comprend des crevettes, des huîtres ou du poisson.
21. Utilisation d'une huile d'unicellulaire telle que revendiquée dans l'une quelconque des revendications 1 à 3 ou telle que produite par une méthode telle que revendiquée dans l'une quelconque des revendications 4 à 12, dans la préparation d'un produit alimentaire, d'un produit convenant à l'apport de nutrition parentérale totale ou d'un produit convenant à un supplément diététique.
22. Utilisation telle que revendiquée dans la revendication 21, dans laquelle le produit alimentaire est une formule pour nourrisson, un aliment pour bébé, ou un supplément diététique.

EP 0 515 460 B1

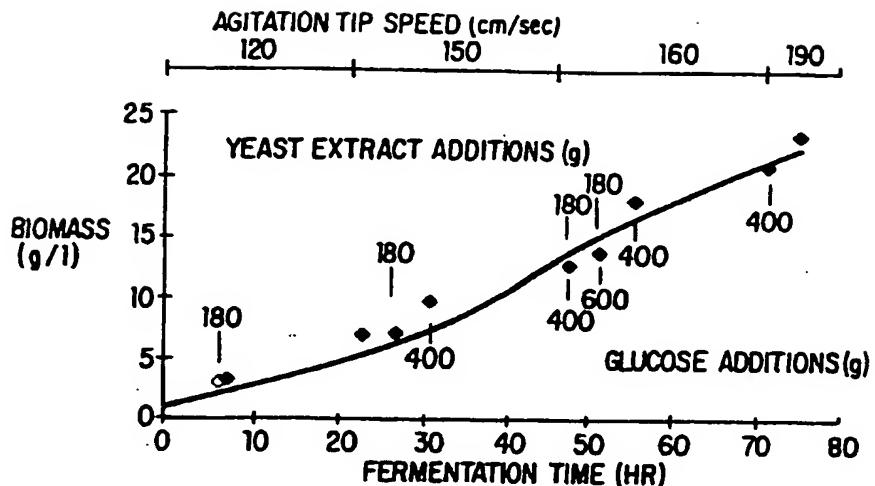


FIG. 1

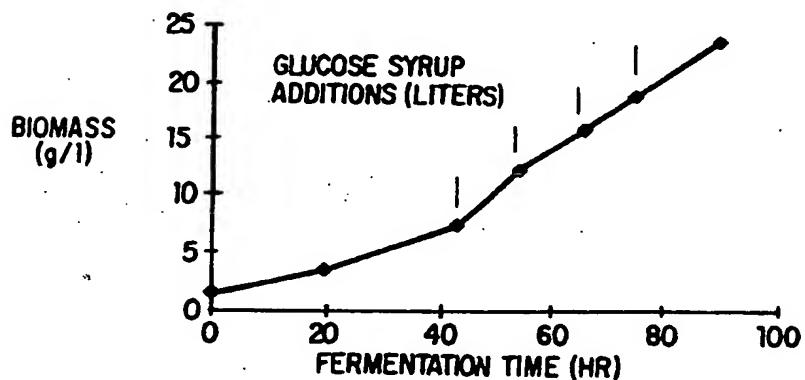


FIG. 2

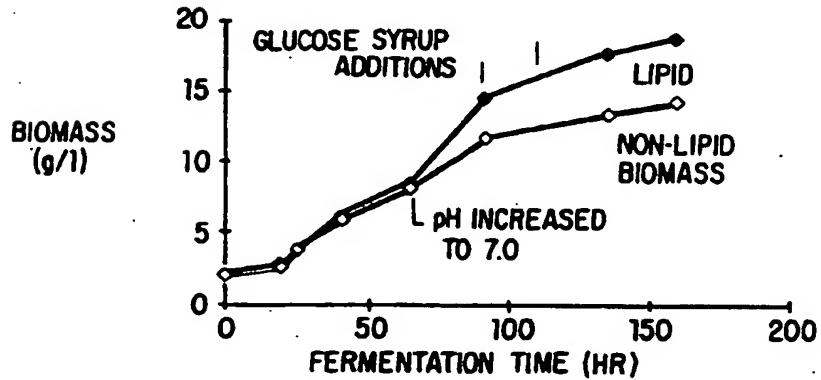


FIG. 3